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DEVELOPMENT OF NEONATAL IMMUNIZATION STRATEGIES USING CpG MOTIFS AS IMMUNE ADJUVANTS

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Development of neonatal immunization strategies using CpG motifs as immune adjuvants

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ABSTRACT

The overall objective is to develop neonatal immunization methods by incorporating immunostimulatory CpG motifs into DNA and protein vaccines. Results showed that three day-old lambs were effectively vaccinated with plasmid encoding tgD or with tgD formulated with Emulsigenand CpG ODN, even in the presence of maternal antibodies. In contrast, tgD formulated with Emulsigen or Emulsigen and non-CpG ODN induced lower humoral and no cellular immune response, so the immunostimulatory activity was due to CpG motifs. Furthermore, a heterologous DNA prime-boost was effective at inducing both strong cellular and humoral immune responses. Therefore, DNA immunization or immunization with prootein formulated with Emulsigen and CpG ODN is an effective strategy to vaccinate neonates that possess high levels of maternal antibodies.

SUMMARY

Neonates generally display low immune responsiveness to conventional vaccines, which may be due to the immaturity of their immune system and interference by maternal antibodies. The overall objective of this project is to develop neonatal immunization methods by incorporating immunostimulatory CpG motifs into DNA and protein vaccines. When we incorporated CpG motifs in the backbone of pMASIA-tgD, a plasmid encoding tgD of bovine herpesvirus-1, we did not observe any effects. In contrast, addition of CpG ODNs to a tgD protein formulated with a commercial adjuvant, Emulsigen, significantly enhanced the humoral and cellular immune responses induced in neonatal lambs. This effect was due to the presence of CpG motifs, since non-CpG ODN only had a moderate effect on the immune responses induced by tgD and Emulsigen. Furthermore, pMASIA-tgD and tgD with Emulsigen and CpG ODN both induced an immune response in newborn lambs with maternal antibodies. A regimen of priming with pMASIAtgD and boosting with tgD formulated with Emulsigen and CpG ODN, induced the strongest immune responses. Since the newborn lambs developed antibody, as well as T cell responses, this clearly confirms the ability of the animals to respond to vaccination at this age. Furthermore, lambs born to tgD-hyper-immunized ewes, thus containing high levels of passively acquired serum antibodies, responded to the tgD DNA and protein vaccines, which shows that the maternal antibodies did not inhibit the development of an immune response. All of the lambs also developed a memory response after secondary immunization. These results indicate that DNA immunization or immunization with protein formulated with Emulsigen and CpG ODN is an effective strategy to vaccinate neonates that possess high levels of maternal antibodies.

Background

In view of the high incidence of infectious disease in young animals, it is obvious that there is a definite need for effective vaccines. However, as their immune system is functionally immature, newborns are not only prone to infections, they also develop weak immune responses to most conventional vaccines, and they specifically appear to have difficulty mounting a cellular, or Th1-type, response, which is essential for successful vaccination against numerous infectious agents, for example bovine respiratory syncytial virus. Even in species that have a substantially developed thymus and lymphoid organs before birth, such as cattle, non-specific defence mechanisms, as well as specific recognition of antigens by B and T lymphocytes, are not as well developed in neonates as in adults [1]. In addition to inducing non-protective immune responses in neonates, ineffective vaccines may even lead to immunopathologic responses. Furthermore, the transfer of maternal antibodies from the mother to the neonate through the colostrum or *in utero* generally provides short-term protection, but at the same time can prevent the development of effective active immunity. Since the levels of maternal antibodies decline at different rates, animals may either be vaccinated too late, which may result in a window of susceptibility to disease, or too early, in which case vaccination may not be effective. Unfortunately neither modified-live vaccines, nor killed or subunit vaccines, are effective in the presence of maternal antibodies.

Approaches

The recent observation that the immune system of vertebrates recognizes unmethylated CpG deoxynucleotides in bacterial DNA as a danger signal has provided us with novel strategies to induce protective immunity in neonates. These DNA sequences, also called CpG motifs, are powerful immunostimulants. Importantly, when the biological activity of such CpG motifs was tested in the form of synthetic CpG oligodeoxynucleotides (CpG ODNs), they were potent Th1-directed adjuvants [2-5]. Although immune activation in in-bred mice requires a GACGTT motif, we identified a GTCGTT motif to be optimal for out-bred species including sheep, cattle, horses, pigs and chickens. DNA containing such CpG motifs stimulates B cells to proliferate and secrete antibodies, and induces monocytes, macrophages and dendritic cells to secrete a number of cytokines (soluble mediators between immune cells). The tendency of CpG motifs to favor

the Th1-dominated immune response pathway may also explain why DNA vaccines, which contain CpG motifs, also generally induce both antibody and cellular immune responses [6-8].

Because of these characteristics, the use of plasmid DNA containing CpG motifs or proteins adjuvanted with CpG ODN constitutes a promising approach to the development of immunity in neonates. Indeed, immunization of neonatal mice with DNA vaccines has resulted in the induction of both antibody and cellular responses against *Clostridium tetani*, *Plasmodium yoelii*, rabies virus, measles virus, herpes simplex virus and influenza virus. There are also indications that DNA vaccines induce immunity against bovine herpesvirus-1 (BHV-1) in neonatal lambs, pseudorabiesvirus in piglets and influenza virus, hepatitis B virus and HIV in primates [9-18]. Although thus far only a few studies have been carried out on the use of CpG ODN for neonatal immunization, CpG ODNs were capable of enhancing cellular immune responses against hepatitis B antigen in neonatal mice [19].

Objectives

The overall objective of this project is to apply the characteristics of CpG motifs to develop immunization regimens for newborn animals that are effective even in the presence of maternal antibodies. Because of the propensity of CpG motifs to stimulate the immune system and to support the development of balanced humoral and cellular immune responses, the use of DNA vaccines with CpG motifs, as well as protein vaccines formulated with CpG ODN, constitutes a promising approach to the development of immunity in neonates with a relatively immature immune system. To achieve our objective we carried out the following specific aims:

1. Express a protective glycoprotein of BHV-1, tgD, in plasmids with varying CpG motifs and produce and formulate tgD protein.
2. Evaluate CpG-enriched plasmids encoding BHV-1 tgD in newborn lambs.
3. Evaluate plasmid encoding tgD and tgD formulated with Emulsigen and CpG ODN in newborn lambs, using various prime-boost regimens.
4. Assess the effect of prime-boost regimens on the magnitude and type of immune responses induced in lambs with maternal antibodies.
5. Compare primary immune responses induced by plasmid encoding tgD or tgD protein with or without CpG ODN, followed by a conventional tgD protein boost.

Results

1. Express a protective glycoprotein of BHV-1, tgD, in plasmids with varying CpG motifs and produce and formulate tgD protein.

Incorporation of CpG motifs into the plasmid backbone has been shown to enhance immune responses in mice to hepatitis B antigen [20] and in cattle to BHV-1 tgD. Therefore, we modified plasmid pMAS (a gift from Dr. H. Davis, Coley Pharmaceutical Group, Ottawa, Canada) by the introduction of an intron and specific CpG-motifs based on the bovine immunostimulatory sequence (BIS) from ODN 2135 (TCGTCGTTTGTCGTTTTGTCGTT).

1.1. Construction of plasmids with CpG motifs

Two complimentary oligodeoxynucleotides were annealed to form a duplex containing eight CpG-motifs with 5'-protuding ends complementary to the restriction enzyme *Ava*II (Fig. 1a). These duplexes were force-directional cloned by random ligation into the *Ava*II site of pMAS. The number of inserts/plasmid was verified by restriction enzyme digest and sequencing. Plasmids pBIS24 (24 motifs) pBIS40 (40 motifs), pBIS88 (88 motifs) and pBIS160 (160 motifs) were selected and further modified by inserting Intron A downstream of the human cytomegalovirus immediate-early (HCMV IE) promoter. Intron A was isolated from pCAN1 by *Eag*I and *Pst*I (blunted with T4 DNA polymerase) and inserted into the pMAS and the CpG-modified vectors cut with *Eag*I and *Eco*RV to create pMASIA, pBISIA24, pBISIA40, pBISIA88 and pBISIA160. Finally, the gene encoding tgD was isolated from pSLIAtgD by *Bgl*II digestion and inserted into the adapted vectors at the *Bgl*II site to create pMASIA-tgD (Fig. 1b), pBISIA24-tgD, pBISIA40-tgD, pBISIA88-tgD and pBISIA160-tgD.

All constructs were verified by restriction digestion and sequencing. Digests of pMASIA-tgD, pBISIA40-tgD and pBISIA88-tgD are shown in Fig. 1c. Expression of tgD from these vectors was confirmed by transient transfection of COS-7 cells. The ^{35}S-labelled tgD gene product, which is secreted into the culture medium, was visualized by SDS-polyacrylamide gel electrophoresis and autoradiography of immunoprecipitated supernatants. The molecular mass and relative gel mobility (Fig. 1d) of the tgD protein was consistent with previous reports. These plasmids were further used for neonatal immunization.

(a)

```
5'-GACTCGTCGT TTGTCGTTTT GTCGTTTCGT CGTTTGTCGT TTTGTCGTTG-3'
   3'-AGCAGCA AACAGCAAAA CAGCAAAGCA GCAAACAGCA AAACAGCAAC CTG-5'
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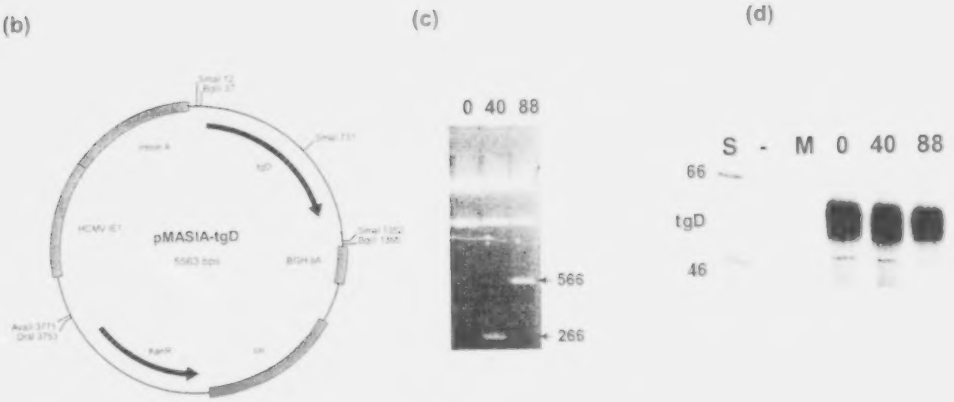

Figure 1. Plasmid construction and verification of tgD expression *in vitro*. (a) Annealed synthetic oligonucleotides used to create CpG inserts. The ends of the 50 bp insert were constructed to permit only unidirectional annealing and ligation into the AvaII site of the vectors. The eight CpG motifs in each insert are bolded and underlined. (b) Plasmid pMASIA-tgD with salient features indicated. The *Ava*II (3773 bp) site was used for inserting the CpG-motifs, which resulted in the reformation of one *Ava*II site. (c) *Ava*II *Dra*I digest of pMASIA-tgD (0), pBISIA40-tgD (40), and pBISIA88-tgD (88). The size of the insert in each plasmid is indicated. (d) Radioimmunoprecipitation of plasmid-encoded tgD from supernatants of transiently transfected COS-7 cell cultures. S, molecular mass standards (kDa); -, mock infected cells; M, pMASIA infected cells; other lane designations are as in c).

1.2. Production and purification of plasmid and protein

Plasmid DNA for immunization was purified from *Escherichia coli* strain DH5α using anion exchange columns, treated to remove endotoxin by repeated Triton X-114 extraction, resuspended at 2.0 mg/ml in sterile endotoxin-free water, and stored at –20 °C. Endotoxin levels in DNA stocks were verified to <0.10 EU/mg DNA (<10 pg/mg DNA), using the *Limulus* amoebocyte lysate QLC-1000 kit.

A truncated secreted version of glycoprotein D (tgD) was produced in transformed Madin Darby bovine kidney cells under regulation of the bovine heat shock 70A (hsp70) gene promoter. The supernatant containing the secreted tgD was collected and purified by size exclusion and ion exchange chromatography. The purified tgD was quantified using a Biorad protein assay and the purity of the protein was established by SDS-polyacrylamide gel electrophoresis. This protein was formulated with CpG ODN, non-CpG ODN and Emulsigen and used to vaccinate newborn lambs. Emulsigen was chosen as co-adjuvant with CpG ODN based on previous observations in adult sheep and cattle [2,3], which showed a synergistic effect between these two compounds.

2. Evaluate CpG-enriched plasmids encoding BHV-1 tgD in newborn lambs.

To evaluate the effect of adding CpG motifs into the plasmid backbone three day-old Suffolk lambs were randomized and allocated to six groups of five lambs each. They were immunized twice intradermally (ID) with a nine-week interval, with 500 µg one of the following plasmids 1) pMASIA-tgD; 2) pBISIA24-tgD; 3) pBISIA40-tgD; 4) pBISIA88-tgD or 5) pBISIA160-tgD. A sixth group was immunized with saline.

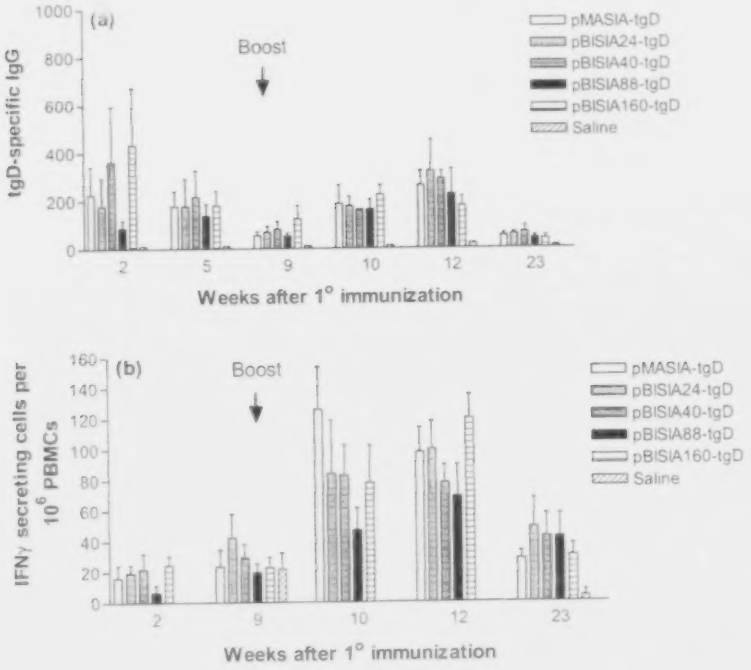

Figure 2. Immune responses induced by CpG enriched plasmids encoding tgD **(a)** Geometric mean tgD-specific ELISA titers were determined in serum and expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the control value. **(b)** The number of tgD induced IFNγ-secreting cells was measured following *in vitro* stimulation of PBMCs with 1 µg/ml of gD. The results represent the average of triplicate wells for each animal. The mean number of IFNγ-secreting cells/10^6 cells for each group of animals is shown. Bars show the standard error of the means.

After one immunization, all but the pBISIA88-tgD immunized group developed significantly higher tgD-specific antibody titers than the saline group ($p < 0.05$) (Fig. 2a). After the second immunization, all of the vaccinated groups had higher antibody titers than the saline group ($p < 0.05$), but there was no difference between the groups. With respect to the cellular response, all

of the plasmid vaccinated groups, except the pBISIA88-tgD group, had significantly higher numbers of IFNγ secreting cells in the peripheral blood mononuclear cells (PBMCs) than the saline group after primary immunization ($p < 0.05$) (Fig. 2b). After the second immunization, all plasmid vaccinated groups had IFNγ secreting cells, and there were no significant differences between these groups. These data indicate that incorporation of CpG motifs does not influence the immune response in newborn lambs, so we continued to use primarily pMASIA-tgD.

3. Evaluate plasmid encoding tgD and tgD formulated with Emulsigen and CpG ODN in newborn lambs, using various prime-boost regimens.

As an approach to optimize the vaccination regimen, we subsequently tested various prime-boost combinations. Four groups of five lambs each were immunized twice with a nine-week interval with one of the following: 1) twice pMASIA-tgD; 2) twice tgD formulated with Emulsigen and CpG ODN (Em/CpG); 3) pMASIA-tgD followed by tgD formulated with Emulsigen and CpG ODN; 4) tgD formulated with Emulsigen and CpG ODN followed by pMASIA-tgD; or 5) saline. pMASIA-tgD was administered at 500 µg/dose ID in 500 µl saline. Truncated gD was delivered at 20 µg/dose, with 30% Emulsigen and 100 µg CpG ODN 2007 (5'-TCGTCGTTGTCGTTTTGTCGTT-3'), SC in 1 ml.

After one immunization, all groups developed an antibody response and there were no differences between the groups. After the second immunization, the group that was first immunized with pMASIAtgD and then with tgD + Em/CpG developed significantly stronger antibody responses than the saline group, as well as all other groups (Fig. 3a) ($p < 0.01$). All groups had significantly higher numbers of IFNγ secreting cells in the PBMCs than the saline group after the first and second immunization (Fig. 3b). The animals immunized with either pMASIA-tgD or plasmid-prime-protein boost had higher numbers of IFNγ secreting cells than the other three groups ($p < 0.05$). These results demonstrate that priming with a tgD DNA vaccine, followed by boosting with tgD protein formulated with Emulsigen and CpG ODN induces better immune responses than any of the other vaccination approaches, because with this vaccination regime both antibody and cellular immune responses were superior.

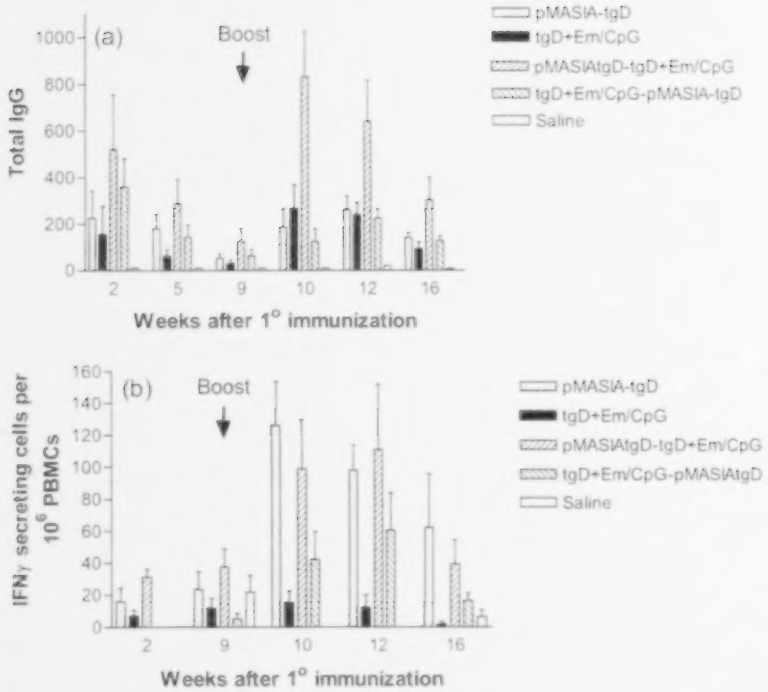

Figure 3. Immune responses induced by pMASIAtgD, tgD formulated with CpG ODN and Emulsigen, plasmid prime-protein boost or protein prime-plasmid boost. **(a)** Geometric mean tgD-specific ELISA titers were determined in serum and expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the control value. **(b)** The number of tgD induced IFNγ-secreting cells was measured following *in vitro* stimulation of PBMCs with 1 μg/ml of gD. The results represent the average of triplicate wells for each animal. The mean number of IFNγ-secreting cells/10^6 cells for each group of animals is shown. Bars show the standard error of the means.

4. Assess the effect of prime-boost regimens on the magnitude and type of immune responses induced in lambs with maternal antibodies.

Since maternal antibodies may interfere with the efficacy of most vaccines in newborn animals, we then tested which of the vaccination approaches would be most effective in lambs with maternal antibodies.

4.1. Vaccination of ewes

Fifty-three pregnant ewes were allocated to two groups. Twenty-eight ewes were immunized with tgD formulated in VSA3, seven and three weeks before lambing, and 25 ewes

were not vaccinated. All ewes were bled at the times of vaccination and one week after the second vaccination. All vaccinated ewes sero-converted to tgD, whereas the non-vaccinated ewes did not have tgD-specific antibodies (Fig. 4).

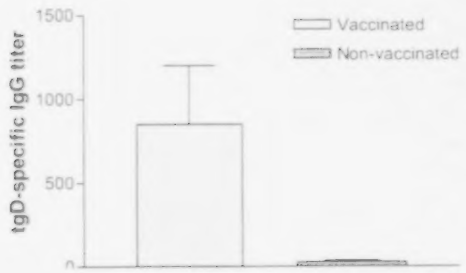

Figure 4. Truncated gD-specific IgG titers in hyper-immunized and non-immunized ewes one week after the second immunization. Geometric mean tgD-specific ELISA titers were determined in serum and expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the control value. Bars show the standard error of the means.

4.2. Immune responses induced in lambs born to tgD hyperimmune and non-immune ewes

The 25 lambs born to tgD hyperimmune and non-immune ewes were randomized and allocated to groups of five lambs each. They were immunized as follows:

Table 1. Vaccination schedule.

Group	Prime	Boost
- maternal antibodies		
1	Saline	Saline
2	pMASIA-tgD	pMASIA-tgD
3	pBISIA160-tgD	pBISIA160-tgD
4	pMASIA-tgD	tgD + Emulsigen + CpG
5	tgD + Emulsigen + CpG	tgD + Emulsigen + CpG
+ maternal antibodies		
6	Saline	Saline
7	pMASIA-tgD	pMASIA-tgD
8	pBISIA160-tgD	pBISIA160-tgD
9	pMASIA-tgD	tgD + Emulsigen + CpG
10	tgD + Emulsigen + CpG	tgD + Emulsigen + CpG

- pMASIA-tgD and pBISIA160-tgD @ 500 µg/dose ID in saline in 500 µl
- tgD @ 20 µg/dose SC in 1 ml, formulated with Emulsigen @ 30% and CpG ODN 2007 (5'-TCGTCGTTGTCGTTTTGTCGTT-3') @ 100 µg/dose
- boost at 15 weeks

Antibody titers specific for tgD were determined by ELISA. Groups 1 to 5 did not have tgD-specific antibodies prior to vaccination, as expected (Fig. 5a). Following immunization with plasmid encoding tgD or tgD protein, these lambs developed low antibody titers. There was a significant difference between the pMASIAtgD vaccinated lambs and the animals immunized with saline ($p < 0.05$). The lambs born to the hyper-immune ewes (groups 6 to 10) acquired passive antibodies through the colostrum and, accordingly, had tgD-specific serum antibody titers three days after birth (Fig. 5b), which gradually declined over a period of 15 weeks. Because of the presence of the maternal antibodies, it was not possible to determine whether active antibody responses had developed. At week 15, all antibody titers had decreased to background levels. After the boost all vaccinated animals, with exception of one animal in the tgD protein group without maternal antibodies, developed tgD-specific antibody titers ($p < 0.05$). There was no effect of maternal antibodies on the groups vaccinated with either tgD DNA vaccine or tgD DNA vaccine followed by tgD protein vaccine. However, the lambs born to non-immune ewes and vaccinated with tgD protein vaccine had higher titers than the animals born to

hyper-immune ewes two weeks after the boost ($p < 0.05$). This may not be significant in the long term, because by week 21 all vaccinated groups had similar antibody titers.

The numbers of tgD-induced IFNγ producing T cells in the PBMCs were assessed in an ELISPOT assay. The majority of the lambs also developed significant numbers of IFNγ secreting cells in the PBMCs after primary immunization, which suggests that they have mounted a cellular immune response as well. There were no significant differences between the vaccine groups. However, two weeks after primary immunization, the lambs that were born to tgD hyper-immune ewes tended to have more IFNγ secreting cells in the PBMCs than the lambs born to non-immune ewes when vaccinated with plasmid encoding tgD (Fig. 5c and 5d). The reason for this is not clear. After the secondary immunization, again most animals responded, confirming that they are capable of mounting a response.

These data confirm that, with exception of one animal, all lambs were able to mount a memory response, so although they were immunized at a very early age, they did not develop tolerance. There also does not appear to be an effect of the presence of maternal antibodies if lambs are vaccinated with DNA vaccine on day 3 after birth, but there may be an effect on the secondary response to the protein vaccine. This would suggest that a DNA prime – protein boost might be the best regimen for vaccinating very young lambs with maternal antibodies.

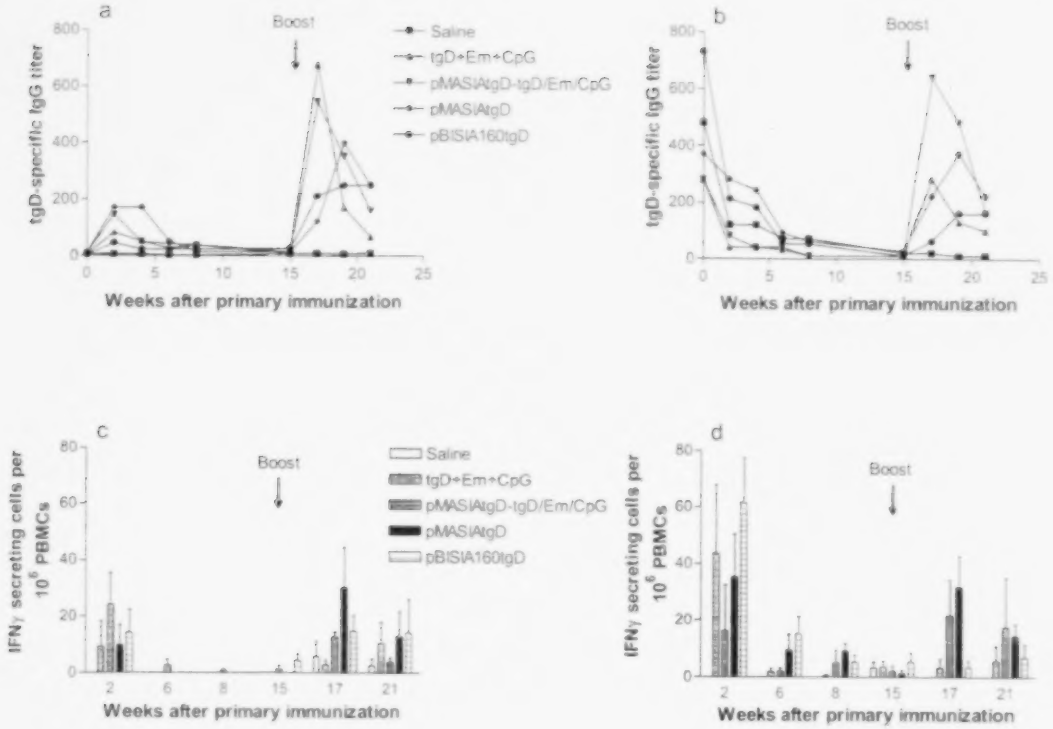

Figure 5. Immune responses induced by plasmid encoding tgD or tgD formulated with CpG ODN and Emulsigen or plasmid prime-protein boost. Three day-old lambs born to either tgD non-immune ewes **(a and c)** or to hyperimmune ewes **(b and d)** were vaccinated either with plasmid encoding tgD or with tgD protein and boosted at 15 weeks of age. **(a and b)** Geometric mean tgD-specific ELISA titers were determined in serum and expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the control value. **(c and d)** The number of tgD induced IFNγ-secreting cells was measured following *in vitro* stimulation of PBMCs with 1 μg/ml of gD. The results represent the average of triplicate wells for each animal. The mean number of IFNγ-secreting cells/10^6 cells for each group of animals is shown. Bars show the standard error of the means.

5. Compare primary immune responses induced by plasmid encoding tgD or tgD protein with or without CpG ODN, followed by a conventional tgD protein boost.

In the previous trials we concluded that a DNA prime-protein/CpG ODN boost is the best approach to induce both cellular and humoral immune response in three day-old lambs. However, based on previous information, we expected that the antibody titers were not at protective levels. Therefore, instead of taking this strategy forward into calves as originally planned, we performed another lamb trial. First, in order to confirm that the adjuvant effect of the CpG ODN was indeed due to the addition of CpG ODN to the formulation, as we observed

for older animals [2], we also tested tgD in combination with Emulsigen alone or with Emulsigen and a stimulatory or non-stimulatory non-CpG ODN. Twenty-five lambs were randomized, allocated to groups of five and immunized with: 1) tgD + Emulsigen; 2) tgD + Emulsigen + CpG ODN; 3) tgD + Emulsigen + non-CpG ODN; 4) pMASIAtgD; or 5) saline. Truncated gD was given SC in 1 ml, at 20 µg/dose with Emulsigen at 30% and CpG ODN 2007 (5'-CGTCGTTGTCGTTTTGTCGTT-3') or non-CpG ODN 2007 (5'-TCGTGCTTGTGCTTTT GTGCTT-3') at 100 µg/dose. pMASIA-tgD was administered ID at 500 µg in 500 µl. To directly compare the ability of each vaccine preparation to prime the immune system, we boosted all groups with tgD in an oil-based adjuvant, VSA3, which has been shown to induce high neutralizing antibody titers.

Two weeks after primary immunization all vaccinated lambs developed significantly increased antibody responses compared to the saline group (Fig. 6). Furthermore, the lambs immunized with tgD/Em/CpG developed significantly higher titers than the saline, tgD and tgD/Em/non-CpG groups two weeks after the primary immunization ($p < 0.05$), and the pMASIA-tgD immunized group had higher titers than the saline, tgD and tgD/Em/non-CpG groups at four weeks ($p < 0.05$). After the boost with tgD-VSA3, all groups showed an anamnestic response, but the tgD/Em/CpG group had significantly higher titers than all other groups ($p < 0.05$).

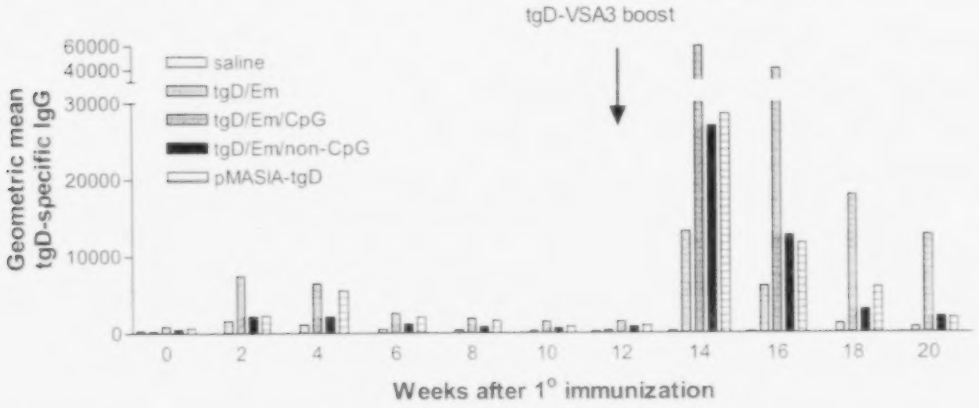

Figure 6. Antibody responses induced by pMASIAtgD or tgD formulated with Emulsigen, CpG ODN and Emulsigen or non-CpG ODN and Emulsigen. Three day-old lambs were vaccinated either with plasmid encoding tgD or with tgD protein in different formulations and they were all boosted at 12 weeks of age with the same vaccine formulation, tgD with VSA3. Geometric mean tgD-specific ELISA titers were determined in serum and expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the control value.

To further evaluate the biological activity of the tgD-specific antibodies, we performed virus neutralization assays. Although all vaccine groups developed virus neutralizing antibodies, both after primary and secondary immunization, the virus neutralization titers were highest in the tgD/Em/CpG group (Fig. 7), which confirms the ELISA data and suggests that these vaccination regimens may lead to protective immunity [2].

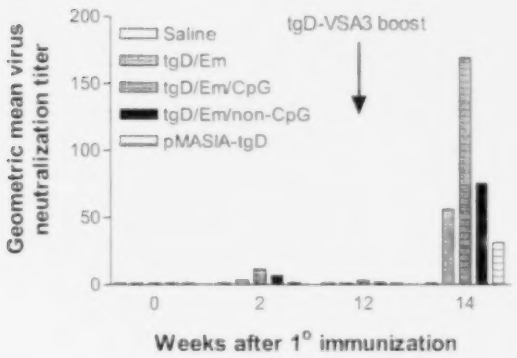

Figure 7. Virus neutralizing antibody responses induced by pMASIA-tgD or tgD formulated with Emulsigen, CpG ODN and Emulsigen, or non-CpG ODN and Emulsigen. Three day-old lambs were vaccinated either with plasmid encoding tgD or with tgD protein in different formulations and they were all boosted at 12 weeks of age with the same vaccine formulation, tgD with VSA3. Geometric mean virus neutralization titers were determined in serum.

As a measurement of cellular immune responses, we evaluated lymphocyte proliferation in the PBMCs of peripheral blood. This assay measures activation of both B and T lymphocytes. After primary immunization, the groups vaccinated with tgD/Em/CpG or with pMASIA-tgD developed proliferating lymphocytes (Fig. 8). After secondary immunization, the tgD/Em/CpG group developed the highest number of proliferating lymphocytes. These levels correlate well with the antibody responses suggesting that at least a portion of the activated lymphocytes are B cells. The presence of antigen-specific T cells in the vaccinated lambs was further analyzed by measuring the number of IFNγ secreting cells in PBMCs isolated from blood at different time points after immunization (Fig. 9). Although there was variation in the responses, which is due to the outbred nature of the animals and the variability of immune cells circulating in the blood at a given point in time, the animals immunized with either pMASIA-tgD or tgD formulated with CpG ODN developed IFN-γ secreting cells, which agrees with the fact that both CpG ODN and

DNA vaccines promote the development of Th1, cellular immunity, whereas Emulsigen or Emulsigen/non-CpG ODN does not. After the boost the cellular immune responses did not increase, which is due to the nature of the VSA3 adjuvant, which, like Emulsigen, promotes the production of a Th2, humoral response.

These data show that the CpG ODN contributed to the previously observed adjuvant effect and that this effect was due to the presence of CpG motifs. Furthermore, after the boost with tgD-VSA3, strong virus neutralizing antibodies were produced.

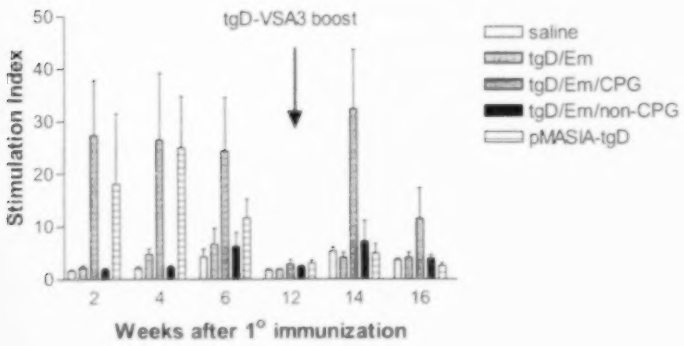

Figure 8. Antigen-specific lymphocyte proliferation following *in vitro* stimulation of PBMCs with 1 µg/ml of gD. The results represent the average of triplicate wells and are expressed as mean stimulation index for each group of animals. Error bars indicate the standard deviation of the means.

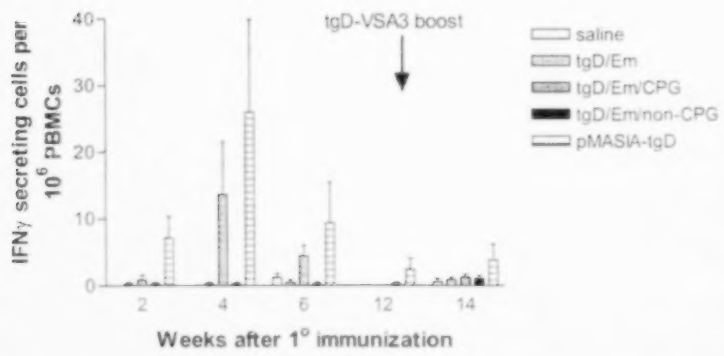

Figure 9. Number of tgD induced IFNγ-secreting cells measured following *in vitro* stimulation of PBMCs with 1 µg/ml of gD. The results represent the average of triplicate wells for each animal. The mean number of IFNγ-secreting cells/10^6 cells for each group of animals is shown. Bars show the standard error of the means.

CONCLUSIONS AND FURTHER DEVELOPMENTS

Since many infections occur very early in life, it is important to be able to vaccinate newborns before the level of maternal antibodies declines to the point where they are no longer protective. However, newborns specifically appear to have difficulty mounting a cellular, or Th1-type, response, so we need to use a vaccine that is a strong inducer of cellular immunity and works in the presence of maternal antibodies. The hypothesis for this project was that CpG motifs, which promote cellular immunity, would enhance the efficacy of DNA and/or protein vaccines in newborn lambs.

Bacterial plasmids used for DNA immunization already have CpG in the backbone. There is evidence in the literature that incorporation of additional, specific immunostimulatory CpG motifs into a plasmid improves the DNA vaccine, whereas mixing CpG ODNs with the plasmid is detrimental due to competition to get into the cells of mice. However, we demonstrated that there is no effect of incorporation into the plasmid backbone on either the cellular or the humoral immune responses induced in newborn lambs. The CpG motif we used is stimulatory in other contexts and we already varied the number of motifs, so in the future we could only modify the spacing of the CpG motifs. Alternatively, we could mix the CpG ODNs with the plasmid, since there are now reports that this may enhance the efficacy of a DNA vaccine, and this may well work in larger animals, where competition between plasmids or plasmid and CpG ODN has not been demonstrated.

However, we further demonstrated that we can effectively vaccinate three day-old lambs with plasmid encoding tgD or with tgD formulated with Emulsigen and CpG ODN, even in the presence of maternal antibodies. In contrast, tgD formulated with Emulsigen or Emulsigen and non-CpG ODN induced lower humoral and no cellular immune responses, so the immunostimulatory activity was due to CpG motifs. This confirms that a DNA vaccine and a protein/CpG vaccine, which are strong inducers of Th1 biased, cellular immunity can both elicit strong and balanced immune responses in newborn lambs. Furthermore, a heterologous DNA prime-protein boost is an effective approach to induce both strong cellular and humoral immune responses, specifically when lambs have maternal antibodies. In future experiments we plan to

further optimize the dose of CpG ODN, which thus far we extrapolated from work in adult sheep.

We had planned to perform a challenge trial in newborn calves using CpG-based vaccination, but decided to postpone this, because we needed to confirm that the responses observed in newborn lambs were due to the presence of CpG ODN and also identify the best approach for priming by using the same vaccine to boost all animals in the trial. However, we already have promising results from a BRSV trial where we vaccinated three week-old calves, which still have an immature immune system and maternal antibodies, with a killed BRSV vaccine formulated with either Emulsigen, Emulsigen and CpG ODN or Emulsigen and non-CpG ODN. Protection from viral challenge was only induced by the CpG ODN formulated vaccine. Thus, we will test pMASIA-tgD and tgD protein formulated with Emulsigen and CpG ODN in a vaccination challenge trial in newborn calves in the near future.

INFORMATION OF BENEFIT TO PRODUCERS, PROCESSORS OR GOVERNMENT

Bovine respiratory disease significantly reduces the profitability of beef production. Infection occurs within days of arrival, affects 14.4% of feedlot placement and causes ~1% mortality. Consequently, estimated losses due to BRD are ~$1 billion annually in North America. These losses are due to both decreased productivity and enhanced treatment and labor cost. To prevent the high incidence of disease, calves should be vaccinated twice and as early as possible. In practical terms, this would happen right after birth or at branding. However, it is difficult to achieve effective protection with the vaccines currently available, because they are not optimized for the relatively immature immune system of young animals or to be effective in the presence of maternal antibodies. The results of this project have shown proof of concept for the potential of two types of vaccines, either a DNA vaccine or a protein – CpG ODN vaccine, to induce immune responses in newborn animals, even those with maternal antibodies. Effective vaccination of newborns would eliminate the window if susceptibility between decline of maternal antibodies and development of immunity. Furthermore, if animals can be vaccinated at branding, and then again upon entry in the feedlot, this would ensure a strong, fast immune response before they become susceptible and exposed to BRD.

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Presentations

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